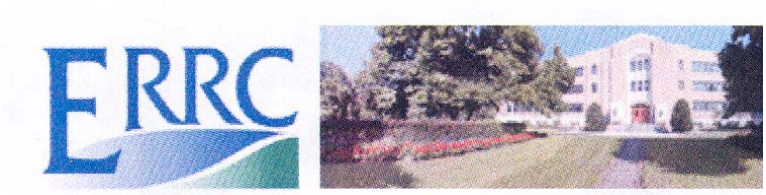

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Title: Isolation of *Salmonella* from Alfalfa Seed and Demonstration of Impaired Growth of Heat-Injured Cells in Seed Homogenates

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Isolation of *Salmonella* from alfalfa seed and demonstration of impaired growth of heat-injured cells in seed homogenates☆

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Abstract

Three major foodborne outbreaks of salmonellosis in 1998 and 1999 were linked to the consumption of raw alfalfa sprouts. In this report, an improved method is described for isolation of *Salmonella* from alfalfa seed lots, which had been implicated in these outbreaks. From each seed lot, eight samples each containing 25 g of seed were tested for the presence of *Salmonella* by the US FDA Bacteriological Analytical Manual (BAM) procedure and by a modified method applying two successive pre-enrichment steps. Depending on the seed lot, one to four out of eight samples tested positive for *Salmonella* by the standard procedure and two to seven out of eight samples tested positive by the modified method. Thus, the use of two consecutive pre-enrichment steps led to a higher detection rate than a single pre-enrichment step. This result indirectly suggested that *Salmonella* cells on contaminated seeds might be injured and failed to fully resuscitate in pre-enrichment broth containing seed components during the first 24 h of incubation. Responses of heat-injured *Salmonella* cells grown in buffered peptone water (BPW) and in three alfalfa seed homogenates were investigated. For preparation of seed homogenates, 25 g of seeds were homogenized in 200 ml of BPW using a laboratory Stomacher and subsequently held at 37 °C for 24 h prior to centrifugation and filtration. While untreated cells grew at about the same rate in BPW and in seed homogenates, heat-injured cells (52 °C, 10 min) required approximately 0.5 to 4.0 h longer to resuscitate in seed homogenates than in BPW. This result suggests that the alfalfa seed components or fermented metabolites from native bacteria hinder the repair and growth of heat-injured cells. This study also shows that an additional pre-enrichment step increases the frequency of isolation of *Salmonella* from naturally contaminated seeds, possibly by alleviating the toxic effect of seed homogenates on repair or growth of injured cells.

Keywords: *Salmonella* isolation; Alfalfa seed; Repeated pre-enrichment; Heat-injury; Repair in seed homogenates

1. Introduction

Alfalfa sprouts have been recognized as an important vehicle for transmission of foodborne illness during the past few years primarily due to contamination with *Salmonella* (National Advisory Committee on Microbiological Criteria for Foods (NACMCF),

☆ Mention of brand or trade name does not constitute an endorsement by the US Department of Agriculture over others of a similar nature not mentioned.

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1999; Taormina et al., 1999). Although epidemiological studies indicated that seeds used for sprouting are the most likely source of contamination (Mahon et al., 1997; Van Beneden et al., 1999), attempts to isolate the pathogen from implicated seeds are not always successful. Furthermore, in cases where isolations were possible, the populations of *Salmonella* detected in contaminated seeds were extremely low, usually in the range of 0.1–1.8 cells per 25 g of seed (Inami et al., 2001; NACMCF, 1999; Stewart et al., 2001). It is presently unclear if *Salmonella* is present at a uniformly low level in contaminated seed lots or if contamination is nonuniform with a few highly contaminated seeds dispersed throughout a lot. It is also possible that *Salmonella* may become severely injured on contaminated seeds during production and storage and requires modification of the existing procedures for isolation.

Conventional methods for isolation of *Salmonella* usually include three procedures, pre-enrichment, selective enrichment, and detection on indicator agar media (Andrews et al., 1995). Several reports (D'Aoust, 1981; Fagerberg and Avens, 1976; Hammack et al., 2001) have shown that use of pre-enrichment increases the frequency of recovering the pathogen from contaminated foods or clinical specimens. However, results from other studies (Fricker, 1987) indicated that use of pre-enrichment did not aid the isolation of *Salmonella* from other types of foods or environmental samples. The need to include pre-enrichment in the isolation scheme appears to be dependent on the physiological state of the pathogen present and the biological and chemical characteristics of specific types of foods or samples to be tested (Fricker, 1987). For samples containing high levels of viable *Salmonella* cells, use of pre-enrichment may not be necessary (Fagerberg and Avens, 1976). However, for samples containing low levels of inactive or potentially injured pathogens (Busta, 1976; Mackey and Derrick, 1982; Sarlin et al., 1998), use of pre-enrichment is expected to be beneficial, if not essential. Such a practice has become a routine procedure for isolating *Salmonella* from alfalfa seeds associated with previous outbreaks (Inami and Moler, 1999, 2001; Stewart et al., 2001; Suslow et al., 2002).

The initial objective of this study was to determine if a modification of pre-enrichment procedures could be developed to improve the isolation of *Salmonella* from naturally contaminated seeds. In the second part of the study, the growth characteristics of heat-injured and untreated *Salmonella* cells in seed homogenates and in fresh pre-enrichment broth were compared.

2. Materials and methods

2.1. Bacterial strains and culture media

A nalidixic acid-resistant strain of *Salmonella* Mbandaka, designated S14, was used for studying the effect of seed homogenates on repair and growth of heat-injured cells. This strain was isolated in our laboratory from naturally contaminated alfalfa seeds implicated in the 1999 Oregon outbreaks (Suslow et al., 2002). Strain S14 was routinely maintained and cultured on Brain Heart Infusion Agar (BHIA, Difco Laboratories., Detroit, MI) supplemented with 20 μg/ml of nalidixic acid at 37 °C. The media used for isolation studies included Buffered Peptone Water No. 2 (BPW), Rappaport-Vassiliadis (RV) R10 Broth, Tetrathionate (TT) Broth, Xylose-Lysine-Tergitol 4 (XLT4) Agar, and semisolid RV (SRV) Agar. All media were obtained from Difco, and prepared according the instructions provided by the manufacturer except SRV, which was prepared in the laboratory by supplementing RV Broth with 0.3% of agar (Difco).

2.2. Sources of alfalfa seeds and preparation of seed homogenates

Six alfalfa seed lots implicated in three sprout-related outbreaks of salmonellosis in 1998 and 1999 were used for isolation. Seed lots 36120, 45197, and CoA98, were provided by Dr. M.L. Tortorello (FDA, Summit-Argo, IL). Seed lots 7166 and 8199a were provided by Dr. A. Charkowsky (USDA, now University of Wisconsin, Madison) and seed lot 8119b was obtained from Dr. T. Suslow (University of California, Davis). Seed lots 45197, 8119a and 8119b were associated with the 1999 outbreaks in Oregon and Washington states (NACMCF, 1999). Seed lots 36120 and 7166 were associated with the 1998 outbreak in California and seed lot CoA98 with the 1999 outbreak in Wisconsin (Proctor et al., 2001). Seed homogenates for studying the effect on repair

and growth of injured cells were prepared from three lots including I-001, 8119b, and 7166. Lot I-001 was not contaminated and was purchased from Caudill Seed (Louisville, KY). Twenty-five grams of seed from each seed lot were submerged in 200 ml of BPW and allowed to soak at 37 °C for 2 h. The pummeling was performed using a Stomacher (Seward Model 400, London, UK) at high speed for 1 min. The stomached samples were incubated at 37 °C for an additional 16 h. After that, the aqueous portion of the sample was collected and centrifuged at 27,000*g* for 30 min to remove the seed and bacterial debris. The supernatant was collected and sterilized by filtration using a Millipore nylon 0.45 μm membrane and the filtrates were stored at 4 °C and later used to grow injured and uninjured cells of strain S14 as described below.

2.3. Isolation of *Salmonella* from naturally contaminated seeds

In each experiment, eight samples (25 g per sample) from each seed lot were tested for the presence of *Salmonella* by the US FDA Bacteriological Analytical Manual (BAM) procedure using XLT4 and SRV as indicator agar media (Andrews et al., 1995) and also by a modified BAM method applying two successive pre-enrichment steps. Detailed procedures of both methods are outlined in Fig. 1. Briefly, seed samples were pre-enriched in BPW once or twice. Pre-enriched cultures were then transferred to selective enrichment broth (TT) and allowed to incubate at 37 °C for 24 h. Fifty-microliter aliquots of selectively enriched cultures were streaked or plated on indicator agar media, XLT4 and SRV. Suspected *Salmonella* isolates forming black colonies on XLT4 and a motility zone on SRV were isolated and further characterized by serological and PCR assays.

2.4. Characterization of *Salmonella* isolates by PCR and serological techniques

Presumptive *Salmonella* colonies were first confirmed by the immunodiffusion method (1–2 Test, BioControl Systems, Bellevue, WA) followed by PCR assay (BAX® System for Screening of *Salmonella*, Qualicon, Wilmington, DE). Typing to somatic antigen group was conducted using commercial anti-

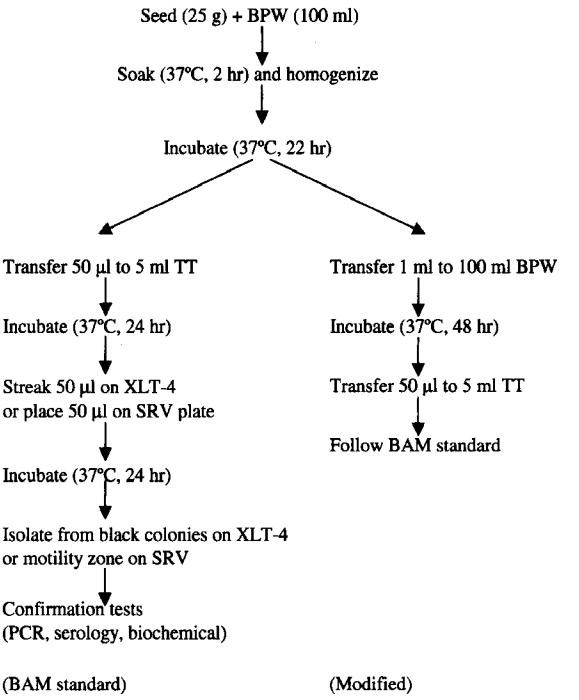

Fig. 1. Procedures for the isolation of *Salmonella* by the standard Bacteriological Analytical Manual (BAM) and a modified BAM method applying two pre-enrichment steps. Note that indicator agar media used are SRV and XLT-4 instead of the agar media recommended in the BAM procedure.

Salmonella antisera (Difco). Two to three confirmed isolates obtained from each seed lot were further characterized to serovars at the National Veterinary Services Laboratories (APHIS, USDA, Ames, IA).

2.5. Induction of heat-injured cells

An overnight culture of *Salmonella* Mbandaka S14 was suspended in phosphate buffered saline (PBS, pH 7.2) to an initial optical density (OD) of 1.0±0.01 at 600 nm. The 250-ml Erlenmeyer flasks each containing 50 ml of PBS were pre-warmed in a water bath (52 °C) for 5 min and subsequently inoculated with 200 μl of the cell suspension. Inoculated flasks were held at 52 °C in the water bath for up to10 min. At 2-min intervals, triplicate flasks were removed and placed on ice. After the 10-min experimental period, samples collected at 2-min intervals were plated on BHIA and BHIA+3% NaCl (designated B-S) and allowed to incubate at 37 °C for 48 h to determine the number of injured and uninjured cells in the sam-

ples. Injured cells were characterized by their inability to grow on B-S. The percentage of injured population in a heat-treated sample was calculated by: [(number of viable cells determined on BHIA−number of viable cells determined on B-S)/number of viable cells determined on BHIA]×100%. The percentage of survival following heat treatment was calculated by: [number of viable cells in a heat-treated sample determined on BHIA/number of viable cells in an untreated sample determined on BHIA]×100%. The survival and injury curves of strain S14 in response to heat treatment were plotted using Microsoft Excel 98.

2.6. Repair and growth of heat-injured cells in BPW and seed homogenates

BPW and seed homogenates (I-001, 8119b, and 7166) at pH in the range of 5.9–6.7 were inoculated with a suspension of heat-injured (52 °C, 10 min) cells of strain S14 to an initial concentration of approximately 10^5–10^6 CFU/ml. BPW and seed homogenates inoculated with an unheated (uninjured) cell suspension at about the same concentration were used as controls. Relative proportions of injured and uninjured cells in heat-treated suspensions were determined by plating the suspension on BHIA and B-S. The changes in the total population of untreated and heated cells of strain S14 grown in BPW and seed homogenates were determined after incubating the cultures at 37 °C for 8 and 16 h.

The lag period of untreated (uninjured) cells in BPW and homogenates was estimated to be 2 h (data not included) and the number of bacterial generations (n) during the 6-h exponential period following the lag phase was calculated according to the formula: $N_0 2^n = N_t$, where N_0=initial cell number (=2 h after incubation) and N_t=cell number after time t (=8 h after incubation). The growth rate (μ) of untreated (uninjured) cells during the 6-h exponential phase was calculated according to the formula: $\mu = t/n$ (min/generation) and was assumed to be the same for injured cells in heated samples grown in the same medium following recovery. The number of generation (n) for uninjured and injured cells in heated samples was equivalent to $360/\mu$ and $(360-x)/\mu$, respectively, where μ was calculated based on the data generated from the study with untreated samples. The extended time (x) required for the injured cells in heated samples to recover in BPW or seed homogenate beyond the 2-h lag period could be calculated based on the formula: [(number of injured cells at N_0)$2^{(360-x)/\mu}$]+[(number of uninjured cells at N_0)$2^{360/\mu}$]=N_t. The numbers of injured and uninjured cells in heated samples at N_0, the total cell number after 6 h of incubation (N_t), and the growth rate (μ) were determined as described above.

3. Results

3.1. Isolation of Salmonella from contaminated seeds

Salmonella was successfully isolated from six alfalfa seed lots implicated in the 1998 and 1999 sprout-related outbreaks of salmonellosis, even though the seed lots had been stored in the laboratory for an additional 2–3 years since the outbreaks. The frequency of isolating *Salmonella* from the seed varied with the seed sources. For example, in the seed lots associated with the 1999 Oregon outbreak (Table 1), one out of eight samples from two seed lots implicated in 1999 Oregon/Washington outbreaks (8199a and 45197) tested positive for the presence of *Salmonella* by a slightly modified BAM procedure (as outlined in Fig. 1) whereas four out of eight samples from seed lot 8199b implicated in the same outbreak tested positive by the same procedure. In contrast, one out of eight samples from each of the two seed lots (36120 and 7166) implicated in the 1998 California outbreak tested positive for the presence of *Salmonella*. Presumptive *Salmonella* isolates obtained from each seed lot were confirmed as *Salmonella* by the serological and PCR tests. Two to three representative isolates obtained from each seed lot were further characterized to serovar by serotyping. The isolates obtained from seed lots 8119a, 8119b, and 45197 implicated in the 1999 Oregon/Washington outbreaks were identified as serotype Mbandaka and the isolates from seed lots 36120 and 7166 implicated in the 1998 California outbreak were identified as serotypes Cubana and Tennessee. Isolates from lot CoA98 implicated in the 1999 Wisconsin outbreak were identified as serotype Muenchen.

The frequencies of recovering *Salmonella* from contaminated seeds also were affected by the isolation method used. The use of the modified method with

Table 1
Isolation of *Salmonella* from naturally contaminated seed lots by the conventional and modified FDA Bacteriological Analytical Manual (BAM) method

Seed sample	Outbreak year	Serotypes identified	Outbreak location	Frequency of positive isolation by		Estimated number of cells/25 g[a]
				BAM	Modified BAM	
36120	1998	Cubana Tennessee	California	1/8[b]	3/8	0.4
7166	1998	Cubana	California	1/8	3/8	0.4
8119a	1999–2001	Mbandaka	Oregon[c]	1/8	2/8	0.3
8119b	1999–2001	Mbandaka	Oregon[c]	4/8	6/8	0.8
45197	1999–2001	Mbandaka	Oregon[c]	1/8	4/8	0.5
CoA98	1999–2002	Muenchen	Wisconsin	5/8	7/8	0.9

[a] Based on the frequency determined by the modified method. A positive sample was assumed to contain one cell in 25 g of seed. Estimated *Salmonella* number=total number of cells in eight samples divided by 8.
[b] Number of positive samples/eight samples tested (25 g/sample).
[c] Also occurred in Washington State.

two pre-enrichment steps led to an increase in detection frequency over the standard method using only a single pre-enrichment step (Table 1). For example, one out of eight samples from seed lots 36120, 7166, or 8119a tested positive by the standard isolation procedure whereas two to four out of the same set of eight samples tested positive by the modified method (Table 1).

Based on the frequency of isolation from eight 25-g samples and assuming a positive sample containing one cell per 25-g seed, the number of *Salmonella* in the contaminated seeds was estimated to be 0.3 to 0.9 cells per 25-g seed. The result presented here suggested that some of the *Salmonella* cells in the contaminated seeds might have been severely injured during seed production or storage and that some of injured cells failed to resuscitate in pre-enrichment broth containing seed homogenates during the first 24 h of incubation.

3.2. Delayed recovery of heat-injured cells in seed homogenates

To determine if pre-enrichment broth containing seed homogenates impaired the recovery and growth of injured cells, a series of experiments were first conducted to establish an adequate condition for inducing injury cells of strain S14 by heat treatment (52 °C

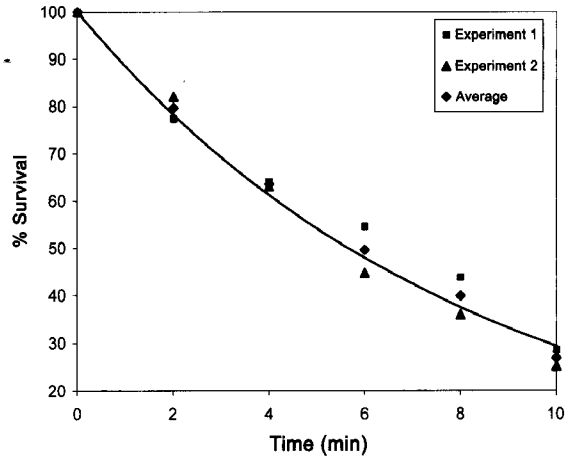

Fig. 2. Survival curve of *Salmonella* Mbandaka S14 heated at 52 °C for up to 10 min.

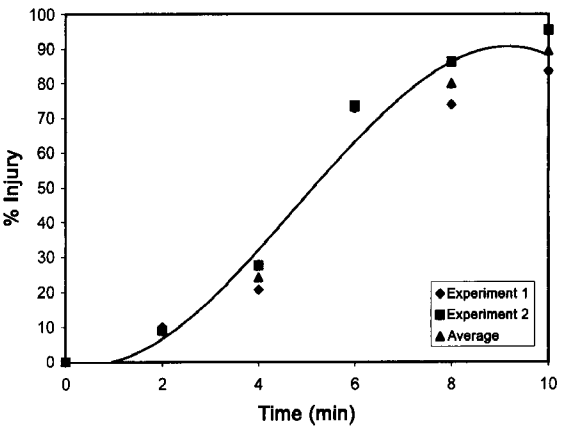

Fig. 3. Injury curve of *Salmonella* Mbandaka S14 heated at 52 °C for up to 10 min.

for up to 10 min). The percent of cells surviving the heat treatment negatively correlated with the duration of treatment (Fig. 2). The proportion of injured and uninjured cells in the samples was determined by plating the heated sample on media BHIA and B-S. A positive correlation between the duration of treatment and the percent of cells injured was observed (Fig. 3). Cell suspensions of strain S14 treated at 52 °C for 10 min were used as the inocula for the experiments described below. This treatment resulted in approximately 30% survival (Fig. 2) and approximately 90% injury among those surviving cells (Fig. 3). A treatment condition resulting in 30% survival has been frequently used to generate injured cells for research in other laboratories (Mackey and Derrick, 1982).

The population changes of strain S14 in homogenates or BPW inoculated with heat-injured or untreated (uninjured) cells were determined after incubation at 37 °C for 8 and 16 h. Results showed that the increase in population of strain S14 grown in BPW and in homogenates inoculated with an untreated (or uninjured) suspension occurred mostly during the first 8 h of incubation (Table 2). An increase of 3.1 log units was observed in BPW whereas an increase of 2.3–2.9 log units was observed in three seed homogenates. Only a slight increase (0.2 to 0.7 log) in BPW or homogenates was observed during the second 8 h of incubation, indicating that the culture almost reached a maximal cell density at 8 h. However, when seed homogenates were inoculated with heat-treated cells, an increase of 0.9–1.9 and 1.8–3.1 log units in the population of strain S14 was observed during the first and the second 8-h of incubation, respectively. While heat-treated and untreated cells grew at about the same rate in BPW, the growth of heat-injured cells in seed homogenates prepared from lot 1766 was at a much slower rate than in BPW. Since the lag period of untreated (or uninjured) cells in BPW and in homogenates had been pre-determined to be approximately 2.0 h, the lag period of heat-injured cells in BPW and in seed homogenates was calculated to be 2.1 and 2.7–5.8 h, respectively. This result implies that heat-injured cells require approximately 0.5 to 4.0 h longer to recover in seed homogenates than in BPW.

4. Discussion

We report here that application of two consecutive pre-enrichment steps improves the recovery of *Salmonella* from naturally contaminated seeds, which had been implicated in the 1998–1999 outbreaks. By using the same procedure, we so far were unable to isolate this pathogen from the seed lot involved in a 1996 international *Salmonella* Stanley outbreak (Mahon et al., 1997) and from a seed lot involved in a recent sprout outbreak in California (August and Brooks, 2001). Although this study showed that *Salmonella* was able to survive in contaminated seeds

Table 2
Comparison of population changes and estimated lag period of heat-injured and uninjured *Salmonella* grown in Buffered Peptone Water (BPW) and in alfalfa seed homogenates[a]

In media or homogenate	Treatment of cells	Initial cell # (log cfu/ml)	Population increase (log cfu/ml)		Final population (log cfu/ml), 16-h incubation	Estimated lag period (min)
			First 8-h incubation	Second 8-h incubation		
BPW	Untreated	5.9[b]	3.1	0.2	9.1	120
	Heat-Injured	5.0	3.0	1.0	8.9	127
I-001	Untreated	5.9	2.7	0.6	9.1	120
	Heat-Injured	5.0	1.9	1.9	8.8	228
8119	Untreated	5.9	2.3	0.7	8.9	120
	Heat-Injured	5.0	2.1	1.4	8.5	160
7166	Untreated	5.9	2.9	0.2	8.9	120
	Heat-Injured	5.0	0.9	2.9	8.8	349

[a] Seed homogenates were prepared as described in Materials and methods. Injured cells were prepared by exposing the *Salmonella* Mbandaka S14 cell suspension to 52 °C for 10 min.

[b] Values represent an average of two experiments, each done in triplicate.

for at least 2–3 years, we also failed to isolate *Salmonella* Newport from seed lot associated with the 1996 outbreak (Inami et al., 2001). Based on the frequency of positive isolation from eight 25-g samples, the number of *Salmonella* in the seed lot implicated in the 1998 and 1999 outbreaks was found to be in the range of 0.3–0.9 cells per 25 g of seed. This estimate is comparable with the most probable number (MPN) that was previously determined (Inami et al., 2001; Proctor et al., 2001; Stewart et al., 2001; Suslow et al., 2002), despite the fact that the seed used in the study has been stored for additional time since the last determination. The responsible *Salmonella* serotypes as identified in this study are consistent with those reported before (Table 3). However, an H_2S-negative strain of serotype Havana known to be associated with the 1998 California outbreak was not detected (NACMCF, 1999). This serotype possibly exists in a relatively smaller proportion as compared to other serotypes (Cubana and Tennessee) and thus appears less frequently on the indicator agars such as SRV. However, this serotype might have been identified if more isolates from seed lots 36120 and 7166 obtained in this study were characterized. Following the characterization of 12 isolates from seed lot CoA98 in our laboratory, we recently identified an additional serotype (Bredeney) in addition to serotype Muenchen in the seed lot CoA98 (Fett, in press).

A combination of two indicator agars such as BGA and XLT4 or HEA has been recommended for isolation of H_2S-positive and H_2S-negative strains of *Salmonella* (Fricker, 1987). This is based on the assumption that H_2S-negative strains of *Salmonella* that are undetectable on XLT4 or HEA will be detected on BGA. In this study, BGA was replaced by SRV based on previous reports that use of SRV improves the isolation of pathogens from food samples possibly containing high levels of competing bacteria (De Medici et al., 1998; Worcman-Barninka et al., 2001). During this study, we found no difference in the effectiveness of using SRV or XLT4 for detection of *Salmonella* in enriched cultures, possibly because the ratio between native bacteria and pathogen in enriched cultures might have reached the level within the detection limit of XLT4.

Inami et al. (2001) has shown that processing seeds by shredding increased the isolation of *Salmonella* from contaminated seeds. It was assumed that shredding the seed enhanced the dislodging of *Salmonella*, which might had firmly attached to the surface or was inside the seeds. The seeds used in this study were presoaked in BPW for 2 h prior to homogenization in a laboratory Stomacher. Although there were no data presented in this study to suggest that this practice improved the recovery of the pathogen from alfalfa seeds as reported previously by Wu et al. (2001), a combination of pre-soaking and homogenization increases the recovery of indigenous bacteria from alfalfa seeds by 1–2 log units (data not shown). In order to increase the frequency of isolating *Salmonella* from contaminated seeds, it is common to use a larger quantity of seed (100 g) per sample as the starting material (Fett, in press; Inami et al., 2001; Suslow et al., 2002) instead of the 25 g per sample described in this study. The correlation between the quantity of the seed

Table 3
Comparison of the pathogen levels and serotypes determined in this study and those reported previously[a]

Outbreak year	Outbreak location	*Salmonella* population (cells/25 g)		*Salmonella* serotypes	
		Estimated in this study	Reported previously	Determined in this study	Reported previously
1995	17 states, Finland	ND[b]	<0.1–0.4	ND	Stanley
1995–1996	7 states, Canada, Denmark	ND	0.1–0.6	ND	Newport, Albany, Schwarzengrund
1998	California and 4 other states	0.4	0.1	Cubana, Tennessee	Cubana, Tennessee, Havana
1999a	Oregon, Washington	0.3–0.8	0.1–1.8	Mbandaka	Mbandaka, Newport
1999b	Wisconsin	0.9	<1.0	Muenchen	Muenchen, Bredeney

[a] From Fett (in press), Inami and Moler (1999), Inami et al. (2001), Proctor et al. (2001), Stewart et al. (2001), and Suslow et al. (2002).
[b] ND=not determined.

used in the sample and the frequency of positive isolation has not yet been carefully examined. The volume ratio between seeds and pre-enrichment broth may be more important than the quantity of seeds itself. Increasing the ratio of seed to volume of BPW is likely to generate more inhibitory homogenates for the repair and growth of injured cells. The ISO method for detection of *Salmonella* in food and animal feeding stuff recommends a ratio (w/v) of 1:10 between foodstuff and pre-enrichment broth.

Presence of antimicrobial factors in processed food samples has been previously indicated (Fagerberg and Avens, 1976). In this study, we further demonstrated that seed homogenates impaired the recovery or growth of heat-injured cells. Since the *Salmonella* cells on naturally contaminated seeds may be injured by desiccation, it needs to be determined if seed homogenates affect the resuscitation of the desiccation-injured cells in the same kinetics as the heat-injured cells as described in this study. Weagant and Bound (2001) and Wu et al. (2001) recently demonstrated that application of immunomagnetic beads improved the detection of *E. coli* O157:H7 in artificially contaminated seeds or sprouts. Use of the immunomagnetic separation technique to eliminate seed constituents from enrichment broths may provide a useful means for preventing their detrimental effect on isolation and detection of *Salmonella* from alfalfa seeds.

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